

Induction of interleukin-6 gene expression by pro-inflammatory cytokines and black-pigmented *Bacteroides* in human pulp cell cultures

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Abstract

Yang L-C, Tsai C-H, Huang F-M, Liu C-M, Lai C-C, Chang Y-C. Induction of interleukin-6 gene expression by pro-inflammatory cytokines and black-pigmented *Bacteroides* in human pulp cell cultures. *International Endodontic Journal*, **36**, 352–357, 2003.

Aim To investigate the effect of pro-inflammatory cytokines and black-pigmented *Bacteroides* on the expression of IL-6 gene in human pulp fibroblasts.

Methodology IL-1 α , tumour necrosis factor- α (TNF- α) and the supernatants of *Porphyromonas endodontalis*, *P. gingivalis* and *Prevotella intermedia* were used to evaluate IL-6 gene expression in human pulp fibroblasts. The levels of mRNAs were measured by the quantitative reverse-transcriptase polymerase chain reaction analysis.

Results Investigations of the time dependence of IL-6 mRNA expression in pro-inflammatory cytokines-treated cells revealed a rapid accumulation of the transcript after 2 h of exposure and remained elevated throughout the 24-h incubation period. In addition, black-pigmented *Bacteroides* also induced IL-6 gene expression in human pulp fibroblasts.

Conclusions Pro-inflammatory cytokines and black-pigmented *Bacteroides* may be involved in developing pulpal inflammation through the stimulation of IL-6 production.

Keywords: black-pigmented *Bacteroides*, IL-6, pro-inflammatory cytokines, pulp fibroblasts.

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Introduction

Dental pulp is a loose mesenchymal tissue almost entirely enclosed in a mineralized tissue, dentine. The most common aspect of pulp injury may be the penetration of bacteria or their surface components into the pulp through a rupture of dentine resulting from either caries or accidental exposure. The end result of the inflammatory process is a necrotic pulp devoid of viable tissue.

A principal driving force behind the pulpal disease response seems to lie in the immune system's response to bacteria, which can enhance the production of inflammatory and bone resorption factors such as leukotriene

B4, prostaglandin (PG), interleukin (IL)-1 α and tumour necrosis factor- α (TNF- α ; Okiji *et al.* 1987, 1989, Sundqvist *et al.* 1995, Tani-Ishii *et al.* 1995). The black-pigmented *Bacteroides* species have gained special attention in the search of aetiologic organisms associated with endodontic infections (van Winkelhoff *et al.* 1988). They are involved in developing pulpal disease through stimulating the production of matrix metalloproteinases (MMPs; Chang *et al.* 2002a), tissue-type plasminogen activator (Yang *et al.* 2003), and inflammatory mediator cyclooxygenase-2 (COX-2) enzyme (Chang *et al.* 2003a) expression in human pulp cell fibroblasts. However, the precise mechanisms of pulpal disease still remain to be elucidated.

The cytokine IL-6 is a major mediator of the host response to tissue injury and infection (Kishimoto 1989, Hirano *et al.* 1990). IL-6 plays a major role in B cell differentiation in the immune system. It is also accepted that IL-6 has multiple biological activities, such as the

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acceleration of bone resorption (Ishimi *et al.* 1990). It is therefore generally recognized that IL-6 is involved in the pathogenesis of various inflammatory diseases such as periodontitis (Takahashi *et al.* 1994). Recently, significant quantities of IL-6 were found to release in the pulp tissues of patients with pulpitis (Barkhordar *et al.* 1999). This indicated that IL-6 derived from human pulp cells may play an important role in the mediation of inflammatory and immune response initiated by oral bacterial infection. However, the relative importance of IL-6 in the pathogenesis of pulpal disease has not been well demonstrated.

In an effort to further define the role of human pulp fibroblasts in the initiation and progression of pulpal disease, the regulation of IL-6 expression by human pulp fibroblasts requires further investigation. In this study, the expression of IL-6 gene in human pulp fibroblasts stimulated by pro-inflammatory cytokine (IL-1 α and TNF- α) and black-pigmented *Bacteroides* (*Porphyromonas endodontalis*, *P. gingivalis* and *Prevotella intermedia*) was investigated by the quantitative reverse-transcriptase polymerase chain reaction analysis.

Materials and methods

All tissue culture biologicals, TRIzol, RNase A, and Moloney murine leukemia virus reverse transcriptase were purchased from Gibco Laboratories (Grand Island, NY, USA). IL-1 α and TNF- α were purchased from Sigma Chemical Co (St. Louis, MO, USA). Cytokines were directly dissolved in the culture medium. The final concentration of IL-1 α and TNF- α used in this study was 10 ng mL⁻¹.

Cell culture

Human pulp fibroblasts were cultured using an explant technique as described previously (Chang *et al.* 1998, Chang & Chou 2001). Briefly, impacted third molars were obtained from healthy patients of the Oral Medicine Center, Chung Shan Medical University Hospital, Taichung, Taiwan. Teeth were sectioned horizontally below the cemento-enamel junction with a number 330 high-speed bur with water spray. The pulp tissue was removed aseptically, rinsed with Hanks' buffered saline solution, and placed in a 60-mm culture dish. Pulp tissue was minced with a blade into small fragments and grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal calf serum (FCS) and antibiotics (100 U mL⁻¹ penicillin, 100 μ g mL⁻¹ streptomycin and 0.25 μ g mL⁻¹ of fungizone). Cultures were maintained at 37 °C in a humidified atmosphere of 5% CO₂ and

95% air. Confluent cells were detached with 0.25% trypsin and 0.05% EDTA for 5 min, and aliquots of separated cells were subcultured. Cell cultures between the third and eighth passages were used in this study.

Bacterial strains and preparation of supernatants

The bacterial strains tested were *P. endodontalis* (ATCC 27067), *P. gingivalis* (ATCC 33277) and *P. intermedia* (ATCC 25611). They were maintained in brain-heart infusion broth prereduced anaerobically, sterilized, and supplemented with 5 mg L⁻¹ haemin and 0.5 mg L⁻¹ menadione. The density of the inoculum, prepared in brain-heart infusion broth, was adjusted to a turbidity of 2 McFarland standard (6×10^8 CFU mL⁻¹). After centrifugation, supernatants were filter-sterilized using a 0.2- μ m filter and stored at -80 °C until used. The supernatants of *P. endodontalis*, *P. gingivalis* and *P. intermedia* were directly diluted in culture medium and the final dilution was 1 : 100.

Treatments

Confluent cells were trypsinized, counted and plated at a concentration of 1×10^5 cells in 60 mm culture dish and allowed to achieve confluence. Cells arrested in G0 by serum deprivation (0.5% FCS for 48 h) were generally used in these experiments. Prior to treatment, the cells were washed with serum-free DMEM and immediately exposed for the indicated incubation times (2, 6 and 24 h) to pro-inflammatory cytokines and the supernatants of black-pigmented *Bacteroides*. Cultures with 0.5% and 10% FCS were used as negative and positive control, respectively.

Total RNA preparation and reverse transcriptase polymerase chain reaction (RT-PCR)

Total RNA was prepared using TRIzol reagent following the manufacturer's instructions. Single-stranded DNA was synthesized from RNA in a 15- μ L reaction mixture containing 100 mg random hexamer and 200 units of Moloney murine leukemia virus reverse transcriptase. The reaction mixture was diluted with 20 μ L of water, and 3 μ L of the diluted reaction mixture was used for the polymerase chain reaction (PCR). PCR reaction mixture contains 10 pmol of forward and reverse primers and 2 units of Tag DNA polymerase. Amplification was performed at 25 cycles for glyceraldehydes-3-phosphate dehydrogenase (GAPDH) and 30 cycles for IL-6 in a thermal cycle. Each cycle consisted of 1 min of denaturation

Gene	Sequence	PCR product (bp)
(A) GAPDH	Forward: 5'-TCCTCTGACTTCAACAGCGACACC-3' Reverse: 5'-TCTCTCTTCCTCTTGTGCTCTTGG-3'	207
(B) IL-6	Forward: 5'-CCACTCACCTCTTCAGAA-3' Reverse: 5'-GCGCAGAATGAGATGAGT-3'	453

Table 1 Nucleotide sequence and size of the expected PCR products for oligonucleotide primers used for RT-PCR

at 94 °C, 1 min of annealing at 57 °C and 1 min of extension at 72 °C. The sequences of primers employed are listed in Table 1. The PCR products were analyzed by agarose gel electrophoresis.

When the cells were probed for IL-6 mRNA production by RT-PCR, a 453-bp band for IL-6 was noted. These bands were consistent with the size as designed by primers. When the band densities were measured and compared with the density of the band obtained for the housekeeping gene GAPDH, relative proportions of mRNA synthesis could be determined within each experiment. The intensity of each band after normalization with GAPDH mRNA was quantified by the photographed gels with a densitometer (AlphaImager 2000; Alpha Innotech, San Leandro, CA, USA). Each densitometric value, expressed as the mean $\pm$ SD, was obtained from three independent experiments.

Results

Investigations of the time dependence of IL-6 mRNA expression in pro-inflammatory cytokines-treated cells revealed a rapid accumulation of the transcript after 2 h of exposure that remained elevated throughout the 24-h incubation period (Fig. 1). The levels of the IL-6 mRNAs increased about 3.6-, 5.1- and 1.7-fold after exposure to IL-1 α for 2, 6 and 24 h, respectively (Fig. 2).

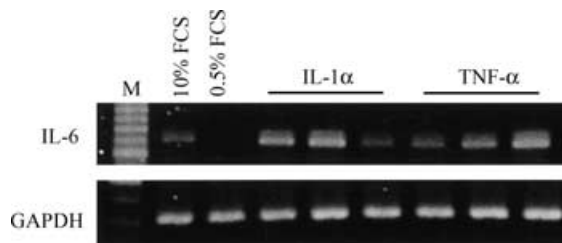


Figure 1 Expression of IL-6 mRNA gene in pro-inflammatory cytokines-treated human pulp fibroblasts by RT-PCR assays. M, DNA molecular size marker. Lanes 1 and 2 represent controls: 0.5 and 10% FCS. Lanes 3–5 represent IL-1 α 2, 6 and 24 h, respectively. Lanes 6–8 represent TNF- α : 2, 6 and 24 h, respectively. GAPDH gene was performed in order to monitor equal RNA loading.

The levels of the IL-6 mRNAs increased about 2.2-, 3.4- and 5.0-fold after exposure to TNF- α for 2, 6 and 24 h, respectively (Fig. 2). However, cells resting in 0.5% FCS did not express detectable levels of IL-6.

To examine the effect of black-pigmented *Bacteroides* on the expression of IL-6 gene, human pulp fibroblasts were treated with *P. endodontalis*, *P. gingivalis* and *P. intermedia*, respectively. Cells resting in 0.5% FCS did not express any detectable level of IL-6 genes. *P. endodontalis*, *P. gingivalis* and *P. intermedia* were found to induce IL-6 gene expression in human pulp fibroblasts (Fig. 3). Densitometric analysis of the IL-6 mRNA gene expression, after normalization by GAPDH, demonstrated that IL-6 mRNAs increased about 3.3-, 2.2- and 2.0-fold after exposure to *P. endodontalis* for 2, 6 and 24 h, respectively (Fig. 4). The levels of the IL-6 mRNAs increased about 1.5-, 3.0- and 1.9-fold after exposure to *P. gingivalis* for 2, 6 and 24 h, respectively (Fig. 4). The levels of the IL-6 mRNAs increased about 2.0-, 1.7- and 1.1-fold after exposure to *P. intermedia* for 2, 6 and 24 h, respectively (Fig. 4).

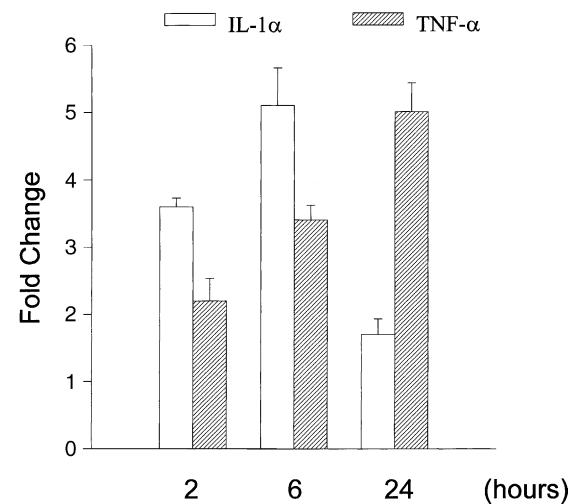


Figure 2 Levels of IL-6 mRNA gene treated with pro-inflammatory cytokines were measured by AlphaImager 2000. Optical density values expressed as the mean $\pm$ SD, were obtained from three independent experiments.

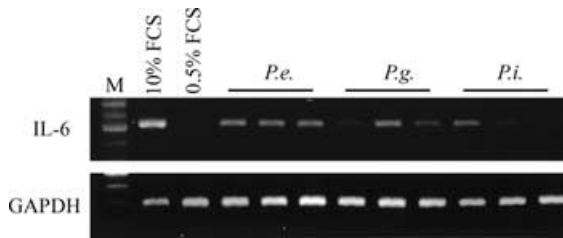


Figure 3 Expression of IL-6 mRNA gene stimulated with black-pigmented *Bacteroides* (*P. endodontalis* (*P.e.*), *P. gingivalis* (*P.g.*) and *P. intermedia* (*P.i.*)) in human pulp fibroblasts by RT-PCR assays. M, DNA molecular size marker. Lanes 1 and 2 represent controls: 0.5% and 10% FCS. Lanes 3–5 represent *P. endodontalis*: 2, 6 and 24 h, respectively. Lanes 6–8 represent *P. gingivalis*: 2, 6 and 24 h, respectively. Lanes 9–11 represent *P. intermedia*: 2, 6 and 24 h, respectively.

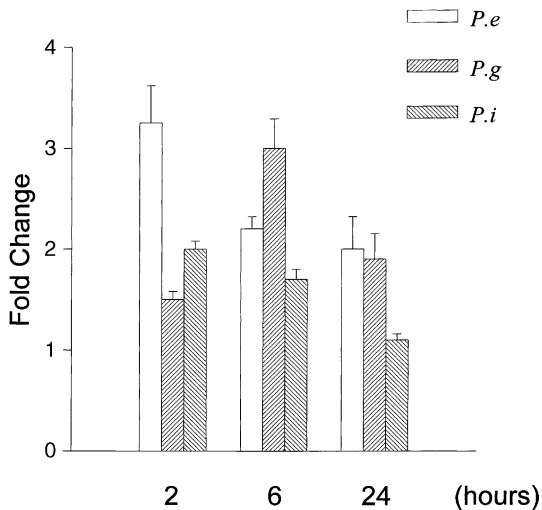


Figure 4 Levels of IL-6 mRNA gene treated with black-pigmented *Bacteroides* were measured by AlphaImager 2000. Optical density values expressed as the mean $\pm$ SD, were obtained from three independent experiments.

Discussion

Cytokine studies have become one of the most important aspects of clinical research. Measurements of cytokine production capability are expected to become new markers for diagnosis or treatment in clinical practice. The RT-PCR is a popular method for analyzing cytokine expression involved in the detection of cytokine mRNA (Kotake *et al.* 1996, Soslau *et al.* 1997). This method is usually applied to the immunological research field,

and the quantitative measurement of cytokine mRNA expression is a useful tool for analyzing cytokine networks. Therefore, we used the RT-PCR method for quantitation of IL-6 mRNA expression in this study.

Recent studies have demonstrated that IL-1 α and TNF- α can activate in human pulp fibroblasts to produce MMPs (Chang *et al.* 2001), tissue-type plasminogen activator (Chang *et al.* 2003b) and COX-2 enzyme (Chang *et al.* 2003c). Black-pigmented *Bacteroides* may result in tissue destruction by activation of one or more distinct host degradative pathways, such as MMPs pathway (Chang *et al.* 2002a) and plasminogen-dependent pathway (Yang *et al.* 2003). Therefore, human pulp fibroblasts can be included in the cytokine network, and these cells may participate in the orchestration of host defence cells in inflamed pulp tissues.

At the site, where inflammation and tissue destruction have occurred, cells might communicate with one another through the interaction of cytokines. Thus, it is important to elucidate completely the complex cytokine cascade flow associated with inflammation-mediated tissue destruction at the molecular level. The present study demonstrated that IL-6 expression by human pulp fibroblasts was enhanced by the pro-inflammatory cytokines, IL-1 α and TNF- α . The induction patterns of IL-1 α and TNF- α were different. The difference in the specific activity could be related either to the type of receptor–molecule interactions or to a different intracellular distribution of second messenger molecules as has been suggested previously (Bauer *et al.* 1988, Katz & Strunk 1989). These possibilities are under investigation at present.

The results of our data are in agreement with Takigawa *et al.* (1994), who reported that IL-6 production by human gingival fibroblasts was enhanced by IL-1 β and TNF- α ; and Lin *et al.* (2002), who demonstrated that obvious induction of IL-6 gene was also noted in pulp fibroblasts after stimulating with IL-1 α and TNF- α . Taken together, it is clearly shown that abundant IL-6 mRNA in human pulp fibroblasts after pro-inflammatory cytokines stimulation suggests the involvement of this cell in the pathogenesis of pulpal disease.

Bacterial components may play an important role in the development of pulpal disease, because pulp tissue injury is a form of connective tissue damage. Such bacterial interference will occur through the dentine, if not exposed. Histological evidence indicates that pulpal disease can be initiated long before bacterial cells reach the pulp tissue, suggesting that inflammatory responses must be elicited from bacterial byproducts rather than the bacteria themselves. Many of the cell components

that have been shown to act as virulence factors in Gram-negative bacteria are associated with the bacterial surface. Lipopolysaccharide (LPS) has been characterized as one such molecule that mediates a number of biological activities leading to the destruction of host tissue. Moreover, LPS is responsible for many; other virulence factors may also contribute to the biological activities exhibited by bacterial byproducts. For this reason, supernatants of bacterial cultures used in this study were used according to recent publications (Chang *et al.* 2002a,b).

Induction of IL-6 gene expression was also noted in pulp fibroblasts after stimulating with black-pigmented *Bacteroides*. This appears to be the first demonstration of IL-6 expression in pulp fibroblasts. Amongst the three supernatants of bacterial cultures tested, the patterns of IL-6 induction were different. *P. endodontalis* was found to be a more potent IL-6 inducer than *P. gingivalis* and *P. intermedia*. The reason for this differential activation is not clear. It may result from a difference in their byproducts. LPS from various sources has been shown to differ in the structure of their lipid A as well as the polysaccharide chain (Novotny 1984).

Consistently, a previous study has shown that *Actinobacillus actinomycetemcomitans*, *P. gingivalis*, and *Escherichia coli* stimulated the IL-6 production in human peripheral blood neutrophils (Euler *et al.* 1998). Taken together, these findings suggest that black-pigmented *Bacteroides* may be involved in developing pulpal disease through the induction of IL-6 production.

In interpreting the results presented here, it is interesting to consider that pro-inflammatory cytokines and black-pigmented *Bacteroides* may lead to the up-regulation of IL-6 production in human pulp fibroblasts. The presence of excessive amounts of IL-6 derived from pulp fibroblasts may influence the inflammatory response by virtue of its ability to activate T and B cells. Therefore, the capacity of human pulp fibroblasts to produce IL-6 in response to pro-inflammatory cytokines and black-pigmented *Bacteroides* suggests that human pulp fibroblasts additionally contribute to the orchestration of immuno-participant cells in the host defence network of pulpal disease. Further studies seem necessary to determine the expression of IL-6 during the inflammatory process of pulpal disease *in vivo*.

Conclusions

The present study demonstrated that IL-6 gene expression by human pulp fibroblasts was induced by the pro-inflammatory cytokines (IL-1 α and TNF- α) and

black-pigmented *Bacteroides* (*P. endodontalis*, *P. gingivalis* and *P. intermedia*). As up-regulation of IL-6 production has been connected with several inflammatory diseases, the results suggest that pro-inflammatory cytokines and black-pigmented *Bacteroides* may be involved in developing pulpal inflammation through the stimulation of IL-6 production.

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